"TGFβ1-INHIBITOR PEPTIDES"

DESCRIPTION OF THE STATE OF THE ART

Cell growth is regulated by various proteins of the growth factor group (Schalch DS et al. (1979) Endocrinology 104:1143-1151). The most important growth factors involved in cell development, and able to act by autocrine and paracrine mechanisms, include the transforming growth factors (TGFs) (Braun L. et al. (1988) Cell Biol. 85:1539-1543; Lyons RM and Moses HL (1990) Eur. J. Biochem. 187:467-473).

The term TGF was first used for describing the activity produced by a cell line transformed with the murine sarcoma virus (deLarco JE and Todaro GJ (1978) Proc. Natl. Acad. Sci. 75:4001-4005; Mizel SB et al. (1980) Proc. Natl. Acad. Sci. 77:2205-2208). The supernatant of these cells was able to induce normal growth, in soft agar, of cells that require a solid support for growth. More specific studies demonstrated two classes of TGF, called TGFα and TGFβ, which in turn comprise families of related proteins. The TGFβ family consists of 5 isoforms (Brand T. and Schneider MD (1995) J. Mol. Cell Cardiol. 27:5-18) of dimeric structure (Schlunneger MP and Grutter MG (1992) Nature 358:430-434; Brand T. and Schneider MD (1995) J. Mol. Cell Cardiol. 27:5-18). Investigations of the mature proteins, purified from a single species, demonstrated a high degree of identity between their sequences (Table 1).

Table 1. Homology among different types of TGFβs. TGFβ1, TGFβ2 and TGFβ3 derived from humans, TGFβ4 derived from chicken and TGFβ5 from frog. (Roberts AB and Sporn MB, 1990).

% of	TGFβ1	TGFβ2	TGFβ3	TGFβ4	TGFβ5
TGFβ1	100				
TGFβ2	71	100			
TGFβ3	72	76	100		
TGFβ4	82	64	71	100	
TGFβ5	76	66	69	72	100

TGFβ1 is synthesized as a precursor of 390 amino acids called Pre-Pro-TGFβ1. In a first hydrolysis there is release of a hydrophobic fragment of 29 amino acids, which gives rise to Pro-TGFβ1. Then the mature TGFβ1 is released by another cut in a region that precedes the terminal amino of TGFβ1 and that consists of two arginines, giving rise to a protein of 112 amino acids with a molecular weight of 12 kDa. To produce the biologically active form, two of these monomers join together by means of disulphide bridges, yielding a dimer of 25 kDa. Alterations of this structure cause loss of biological function (Barnard JA et al. (1990) Biochim. Biophys. Acta 1032:79-87).

Various domains are known to exist within the structure of TGFβ1. One of these domains is found to be located between amino acids 40 and 82 and is involved in the binding of TGFβ1 to its cell receptors (Quian SW et al. (1992) Proc. Natl. Acad. Sci. 89:6290-6294; Burmester JK et al. (1993) Proc. Natl. Acad. Sci. 90:8628-8632).

Receptors of TGFβ1 and other binding proteins

- Five types of specific receptors for TGFβ1 have been characterized (Cheifetz S et al. (1988) J. Biol. Chem. 263:17225-17228 and López Casillas F. et al. (1991) Cell 67:785-795). These receptors have different affinities for the different types of TGFβ1. Receptors

of type I, II and III are the best understood so far (reviewed in Attisano L et al. (1994) Biochim. Biophys. Acta 1222:71-80; Derynck R. (1994) Trends Biochem. Sci. 19:548-553; Yingling et al. (1995) Biochim. Biophys. Acta 1242:115-136). Type IV receptors have also been described (MacKay K. and Danielpour D. (1991) J. Biol. Chem. 266:9907-9911) and type V (Ichijo H. et al. (1991) J. Biol. Chem. 266:22459-22464). It has also been reported that the transmembrane and cytoplasmic domains of endoglin (Cheifetz S et al. (1993) J. Biol. Chem. 267:19027-19030; Bellón T. et al. (1993) Eur. J. Immunol. 23:2340-2345; Yamashita et al. (1995) J. Biol. Chem. 269:1995-2001; Zhang H. et al. (1996) J. Immunol. 156:564-573)) have approximately 70% similarity with the type III receptors, both human and of the rat.

RIII would be the one with the task of binding TGFβ1 and presenting it to RII which in its turn would form a complex with RI (Yamashita et al. (1994) J. Biol. Chem. 269:20172-20178) or to complexes in which various molecules of RI are combined with RII (Weiss G. and Massagué J. (1996) EMBO J 15:276-289). RII-RI interaction would give rise to phosphorylation of RI and subsequent activation of its serine/threonine kinase which would phosphorylate to second messengers like the MADR2 proteins (Macías-Silva M et al., (1996) Cell 87:1215-1224).

(1)

Role of TGFβ1 in hepatic differentiation and regeneration

The effects produced are different depending on the moment of development and on the type of cell.

- Enlargement of the extracellular matrix, on acting upon the liver stellate cells (Ito cells), the principal source of matrix proteins (Mustoe TA et al. (1987) Science 237:1333-1336).

- Differentiation of the epithelial cells and hepatocytes (Florini JR et al. (1986) J. Biol. Chem. 261:16509-16513).
- Inhibition of cell growth during the process of liver regeneration. This effect is of great importance in the maintenance of cell rest *in vivo* (Kato Y et al. (1988) Proc. Natl. Acad. Sci. 85:9552-9556).
- Inhibition of endocytosis of the receptor of the epithelial growth factor (EGF) as has been observed in cultures of foetal rat hepatocytes (Noda M. and Rodan GA (1987) J. Cell Physiol. 133:426-437).

Role of TGFβ1 in hepatic fibrosis

TGFβ1 has been found to be associated with the processes of hepatic fibrosis (Czaja MJ et al. (1989) J. Cell Biol. 108:2477-2482; Annoni G. et al. (1992) J. Hepatol. 14:259-264) causing an increase in production of proteins of the extracellular matrix, by the liver stellate cells (lipocytes or Ito cells), of their receptors and inhibiting synthesis of the proteolytic enzymes that degrade the matrix (Ignotz RA and Massagué J. (1986) J. Biol. Chem. 261:4337-4345). In the liver, TGFβ1 induces the synthesis of collagen and fibronectin in the liver stellate cells (Weiner FR (1990) Hepatology 11:111-117). There is also auto-regulation by increasing its own synthesis, via induction of its mRNA.

TGFβ1 has also been found to be involved in increased synthesis of α2-macroglobulin synthesized by the hepatocytes and the activated liver stellate cells. By binding to TGFβ1 and causing its inactivation (Bachem MG (1994) Ann NY Acad. Sci. 737:421-424), α2-macroglobulin is said to eliminate TGFβ1 from the extracellular compartments.

Investigation of patients with chronic liver damage has shown that there is a correlation between expression of TGFβ1 and expression of the mRNA for the type I procollagen and the serum levels of type III peptide of procollagen (Castilla A. et al. (1991) N. Engl. J. Med. 324:933-940).

Patients with cirrhosis of the liver have a shorter than normal life expectancy owing to the complications that arise in the course of the disease, such as portal hypertension or hepatic failure.

Effect of TGFβ1 on the extracellular matrix

Interaction of TGFβ1 with the cell receptors causes:

- Activation of synthesis of procollagen, fibronectin (Ignotz RA et al. (1987) J. Biol. Chem. 262:6443-6446) and related proteins, including membrane proteins capable of interacting with the components of the extracellular matrix (Carter WG (1982) J. Biol. Chem. 257:13805-13815).
- Inhibition of the synthesis of proteolytic enzymes capable of degrading the matrix (Fukamizu H. and Grinnell F. (1990) Exp. Cell Res. 190:276-282).
- Stimulation of the synthesis of inhibitors of proteolytic enzymes (Fukamizu H. and Grinnell F. (1990) Exp. Cell Res. 190:276-282).

These effects lead to an increase in interactions of the cell with the extracellular matrix, which combined with greater reorganization of the proteins of which it is composed, gives rise to an increase in the total quantity of extracellular matrix (Roberts CJ et al. (1988) J. Biol. Chem. 263:4586-4592). These findings confirm that TGFβ1 is involved in cicatrization processes (Fukamizu H. and Grinnell F.

(1990) Exp. Cell Res. 190:276-282; Barnard JA et al. (1990) Biochim. Biophys. Acta 1032:79-87).

Peptides as inhibitors of ligand-receptor interaction

There is the possibility of using small molecules, synthetic peptides, as analogues of molecules that are present in the body, with the aim of emulating their function. Studies conducted by LeSateur et al. demonstrate the possibility of using cyclized analogues of nerve growth factor (NGF), emulating the β turn region, permitting its binding to the receptor (LeSateur L. et al. (1996) Nature Biotechnology 14:1120-1122). It is also possible to use peptides as antagonists of these molecules, preventing the native factor interacting with its receptor by blocking mediated by the peptide (Lasarte JJ et al. (1994) J. Acquired Immune Deficiency Syndromes 7:129-134; LeSateur et al. (1995) J. Biol. Chem. 270:6564-6569). Earlier studies had demonstrated the usefulness of synthetic peptides as inhibitors of ligand-receptor interaction even when the recognition epitope is not continuous (Daniels AJ et al. (1995) Mol. Pharmacol. 48:425-432). Other studies conducted with the type II receptor of TGFβ1 and with fetuin, a glycoprotein in the group of type II receptors, have demonstrated the possibility of using cyclized peptides as inhibitors of the interaction of TGFβ1 with RII (Demetriou M. et al. (1996) J. Biol. Chem. 271:12755-12761). With this cyclization it becomes possible to obtain peptides with a structure similar to that which could be obtained *in vivo*.

DETAILED DESCRIPTION OF THE INVENTION

For the reasons stated above, we consider that peptides derived both from TGFβ1 and from its receptors, or from proteins with capacity for binding to TGFβ1, could be inhibitors of the action of TGFβ1. We therefore decided to explore this possibility.

Selection of the peptides to be synthesized

The peptides for synthesis were selected in different ways depending on whether they were derived from TGFβ1 or from its receptors.

In the case of the sequence of TGFβ1, peptides were synthesized from 15 amino acids that include the whole sequence of TGFβ1. Each peptide had 10 amino acids in common with its two immediate neighbours.

In the case of the sequences of its receptors, the peptides were chosen on the basis of software designed in our laboratory. One of the computer programs compares two amino acid sequences, with the aim of predicting partially complementary regions. Other programs were also used that were able to predict the regions of the proteins that would be most exposed, on the basis of the hydrophobicity and hydrophilicity of the amino acids making up their sequence.

Synthesis of peptides

The peptides were synthesized by the solid phase method (Merrifield (1963) J. Am. Chem. Soc. 85: 2149-54), using fluorenylmethyloxycarbonyl (Fmoc) as a temporary protecting group of the alpha-amino group (Atherton et al. (1989) Journal of Chemical Society Perkins Transactions 1: 538-546). For the synthesis of

small quantities of a large number of peptides, a multiple synthesizer was used, permitting the simultaneous synthesis of 96 peptides (Borrás-Cuesta et al. (1991) Biologicals 19: 187-190). The peptides were stored at -80°C in the solid state until used.

Purification of the peptides by HPLC

The synthesized peptides were analysed and purified by high-performance liquid chromatography (HPLC), using a Waters 600E-900 system (*Millipore Corp., Bedford, USA*).

A Waters Radial-Pak™ C_{18} 300 Å 15 µm, 8x100mm column (*Millipore Corp., Bedford, USA*) was used for analysis of the peptides by analytical HPLC. The peptide was dissolved in a 0.1% solution of TFA in distilled water, to a maximum concentration of 1 mg/ml. The solution of peptide was injected (100 µl) into the column and was eluted in a water/acetonitrile gradient (Fig. 15) (*Romil Ltd., Cambridge, USA*) both with 0.1% TFA at a flow rate of 1 ml/min. The fractions that contained the peptide were detected by its absorbance at 220 nm and 280 nm (*photodiode array detector, Waters 991, Millipore Corp., Bedford, USA*).

A Waters Delta-Pak™ C_{18} 300 Å 15 µm, 25x100mm column (*Millipore Corp., Bedford, USA*) was used for its purification. The peptide was dissolved and was injected (2 ml) under the same conditions as in the preceding case, employing the same gradient at a flow rate of 5 ml/min. The fraction that contained the pure peptide was collected in a flask.

IN VITRO TESTS. INVESTIGATION OF THE ACTIVITY OF THE PEPTIDES

Cell lines

A line derived from mink pulmonary epithelium, MV-1-Lu, was used (*CCL-64, American Type Cell Culture, Virginia, USA*). The cells were grown in 162 cm^2 culture flasks (*Costar Corporation, Cambridge, USA*) in a stove at 37°C and 5% CO_2, until subconfluence was attained. A complete medium was used: RPMI 1640 with L-glutamine (*GibcoBRL, Life Technologies Ltd., Paisley, Scotland*) supplemented with 5% of foetal calf serum (*FCS, Biological Industries, Kibbutz Beit Haemek, Israel*), 10 mM HEPES (*1M HEPES Buffer, Bio-Whittaker, Verviers, Belgium*) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

Test of inhibition of the growth of the MV-1-Lu cell line

The MV-1-Lu cells grown as indicated above were removed from the bottom of the culture flasks using 5 ml of trypsin-EDTA (*Biological Industries, Kibbutz Beit Haemek, Israel*), resuspended in complete medium and centrifuged at 1500 rev/min for 8 minutes. After centrifugation the cells were resuspended in complete medium to a concentration of 50,000 cells/ml. For conducting the test, 10 ml of the cell suspension were taken and dispensed in 96-well, flat-bottom culture plates (*Costar Corporation, Cambridge, USA*), adding 100 µl/well, and were incubated overnight at 37°C and 5% CO_2, which permits adhesion of the cells to the bottom of the wells. At the end of this time, the peptides to be tested were added in RPMI, to a final

concentration of 200 µg/ml in the presence of a concentration of 200 pg/ml of TGFβ1 in RPMI (*R&D Systems Europe Ltd., Abingdon, UK*). The final concentration of FCS in the well was 2.5%. After 24 hours of incubation, 1 µCi of tritiated thymidine was added per well (*25 Ci/mmol [methyl-^{3}H]-thymidine, Amersham Life Science, Buckinghamshire, UK*) with incubation for a further 12 hours (Grubeck-Loebenstein B. et al. (1989) J. Clin. Invest. 83:764-770; Brennan FM et al. (1990) Clin. Exp. Immunol. 81:278-285).

At the end of the incubation periods the cells were removed from the bottom of the wells with trypsin-EDTA and were collected using a manual harvester (*Titertek cell harvester, Skatron Instruments Inc., Sterling, USA*) which ruptures the cells, collecting the DNA in nitrocellulose filters (*Filter MAT 11731, Skatron Instruments Inc., Sterling, USA*) where it is fixed. The filters were placed individually in 5 ml polypropylene tubes to which 4 ml of scintillation fluid was added (*Biogreen-11, Reactivos Scharlau S.A., Barcelona, Spain*). The activity of each tube was quantified for 90 seconds in a β LKB scintillation counter (*Beta plate system, LKB, Uppsala, Sweden*).

Investigation of inhibition of binding of TGFβ1 to the cell receptors

Selective labelling of the cell receptors (affinity labelling)

The MV-1-Lu cells were removed from the culture flasks incubating them at 37°C for 10 minutes, with 10 ml of solution 1 (128 mM NaCl, 5 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonate at pH 7.5, 5 mM glucose and 1 mM EDTA). The cells thus removed were resuspended in solution 2 (128 Mm NaCl, 5 mM KCl, 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonate

at pH 7.5, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 5 mg/ml BSA) and were collected by centrifugation at 1000 x *g* for 5 minutes. After centrifugation the cells were resuspended in solution 2 at a concentration of 10^6 cells/ml.

From this cell suspension, 0.5 ml aliquots were made in 24-well plates (*Greiner GmbH, Frickenhausen, Germany*), the peptides were added, in 50 µl of a 0.8 mg/ml solution, then this was incubated for 2 hours at 4°C with stirring. Next, ^{125}I-TGFβ1 (2µCi) was added to a final concentration of 277.2 pM (*^{125}I-TGFβ1 human recombinant 800-2200Ci/mmol, Amersham Life Science, Buckinghamshire, UK*) and this was incubated for a further two hours at 4°C with stirring.

After incubation, the cells were transferred to a centrifuge tube and were centrifuged cold at 12,000 x *g* for 1 minute. They were then washed twice in cold solution 2 and were resuspended in 0.5 ml of cold solution 2, 5 µl of dimethyl sulphoxide (*DMSO 99.5%, Sigma Chemical Co., St. Louis, USA*) and disuccimidyl suberate (*DSS, Pierce Chemical Co., Rockford, USA*) giving a final concentration of 0.25 mM of DSS. Reaction was stopped at 15 minutes by dilution, centrifugation and washing with a solution containing 0.25M saccharose, 10 mM Tris and 1 mM EDTA at pH 7.4. The precipitate of cells was resuspended in 0.5 ml of Triton X-100 (*Bio-Rad Laboratories, Hercules, USA*) 1% v/v, 10 mM Tris at pH 7.0, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 1 µg/ml pepstatin and 1 µg/ml leupeptin (*Sigma Chemical Co., St. Louis, USA*) and incubated for 40 minutes at 4°C. The fraction that is insoluble in detergent is separated by centrifugation at 12,000 x *g* for 15 minutes. The fractions that are soluble in detergent (supernatant) and insoluble (precipitate) were frozen at -20°C

(Massagué J. and Like B. (1985) J. Biol. Chem. 260:2636-2645).

Electrophoresis of proteins in sodium polyacrylamide dodecyl sulphate gel

The fractions soluble and insoluble in detergent were used for analysis by electrophoresis in acrylamide/bisacrylamide gels at 7.5% for 5-6 hours at 220 volts.

The proteins were stained with a solution of (*comassie brillant blue*® R250 (*Serva Feinbiochemica GmbH, Heidelberg, Germany*) in methanol (50%), acetic acid (10%) and distilled water, for 30 minutes. Subsequent washings were effected with a solution of methanol (50%), acetic acid (10%) and distilled water for 15 minutes, in the first washing, and methanol (2.5%), acetic acid (0.5%) and distilled water, in the subsequent washings, until the background colour was removed.

Flow cytometry

Inhibition of the binding of TGFβ1, mediated by peptides, to the cell receptors was measured by the direct immunofluorescence method. An immunofluorescence kit was used for this (*Fluorokine rh TGFβ-biotin, R&D Systems Europe Ltd., Abingdon, UK*). This test is based on the capacity of biotinylated TGFβ1 to bind to the cell receptors, in a specific manner, and the subsequent interaction of the biotin with fluorescein-labelled avidin, so that the signal intensity will depend on the quantity of TGFβ1 bound to the cell receptors.

The MV-1-Lu cells grown in 162 cm^2 flasks were removed using solution 1 (described previously) and

were resuspended in physiological saline for centrifugation at 500 x *g* for 5 minutes. After centrifugation, the cells were resuspended again in physiological saline at a concentration of $4x10^6$ cells/ml. 25 µl of the cell suspension was added to 12x75 mm borosilicate tubes, to which was added the peptide to be tested in 40 µl of RPMI 1640 medium, giving a final concentration of 0.42 µg/µl and 10 µl of biotinylated TGFβ1. As a control of specificity, 10 µl of a biotinylated reagent supplied with the kit was added, 10 µl of biotinylated TGFβ1 was added as a positive control and 20 µl of anti-TGFβ1 blocking antibody was added as a negative control. Physiological saline was added to all the controls until a total volume of 75 µl was reached. All the tubes were incubated for 1 hour at 4°C in darkness.

At the end of the incubation period, 10 µl of fluorescein-labelled avidin was added, incubating for 30 minutes at 4°C in darkness, after which 2 ml of a washing solution (RDF1) was added, followed by centrifugation at 500 x *g* for 6 minutes. The cell precipitate was resuspended in 0.2 ml of cold PBS for cytometry (*FACScan, Becton Dickinson Immunocytometry Systems, California, USA*). This method permits measurement of the fluorescence emitted by each cell when a laser beam is incident upon it, by means of a computer program (*Lisys™ II, Becton Dickinson Immunocytometry Systems, California, USA*). Fig. 16 shows a typical image from analysis by flow cytometry.

To obtain the data on inhibition of the binding of TGFβ1 to the receptors, the positive control of the test was used for delimiting the fields corresponding to the labelled cells, that have bound to the TGFβ1-biotin (M2) and to the unlabelled cells (M1). Once the fields had been delimited, the percentage of cells

located in each of them was calculated. The same was done with the data obtained when the peptide was incubated with TGFβ1-biotin or with the cells, depending on whether they were derived from the receptors or the TGFβ1 respectively. With these data, the percentage inhibition of each peptide was calculated using the following formula: 100 - ((M2 Peptide-M2 Negative) x 100 / (M2 Positive-M2 Negative)).

EXPERIMENTS *IN VIVO*. EXPERIMENTAL MODEL OF FIBROSIS

Male white rats (albino Wistar strain) from simultaneous litters (5 weeks ± 1.5 weeks) were used, in order to obtain a group that was homogeneous in age and initial weight. Throughout the experiments, the animals were kept in conditions of constant temperature (22°C) with a 12-hour cycle of light and darkness. They had free access to water and food.

Hepatic cirrhosis (HC) was induced by inhalation of carbon tetrachloride for 11 weeks, twice per week (López Novoa JM et al. (1976) Patología IX:223-240; Camps J. et al. (1987) Gastroenterology 93:498-505). Exposure to CCl_4 was effected by bubbling compressed air, at a flow rate of 3 litres/min, through a gas wash-bottle. One minute of exposure was used initially, increasing by one minute per week until 4 minutes was reached in the fourth week. CCl_4 was not administered during the fifth week, starting again at the sixth week with an exposure of 5 minutes. This exposure time was maintained until week 11. 400 mg/l of phenobarbital (*Luminal®, Bayer, Leverkusen, Germany*) was added to the drinking water, from one week before starting exposure to CCl_4 and until the end of the experimental period. Before starting the treatment, one week was left, in which they were not administered CCl_4. During treatment

they were administered a weekly dose of CCl_4, as recorded (Fig. 2).

Distribution of the animals

The animals were divided into 4 groups before beginning the process of induction of hepatic cirrhosis.

Healthy controls (Co): Animals that were not subjected to the fibrosis process.

Treated healthy controls (Co+P144): Animals that were not subjected to the fibrosis process and that were administered the peptide P144 during the last 3 weeks (coinciding in time with the treatment of the group of rats Tto_2).

Cirrhotic controls 1 (Ci_1): Animals subjected to the process of induction of cirrhosis by inhalation of CCl_4 twice per week. These animals were separated into 2 groups on reaching the fifth week:

Cirrhotic controls 1 (Ci_1): Animals that continued to be subjected to the process of induction of fibrosis up to week 11, without being administered the peptide P144. They were administered saline serum on alternate days, throughout the induction process (weeks 5 to 11).

Treated cirrhotics 1 (Tto_1): Animals that were administered the peptide P144 derived from the sequence of the type III receptor, on alternate days, during the process of induction of fibrosis, from week 5 to week 11.

Cirrhotic controls 2 (Ci_2): Animals that continued to be subjected to the process of induction of fibrosis without receiving the peptide P144 or saline serum. This group was subdivided into another two on reaching week 11.

Cirrhotic controls 2 (Ci_2): Cirrhotic animals that were not subjected to any type of treatment, kept as controls. These animals received injections of saline serum for 3 weeks (weeks 13 to 15).

Treated cirrhotics 2 (Tto_2): Cirrhotic animals that were treated with the peptide derived from the sequence of the type III receptor (P144), for 3 weeks (weeks 13 to 15).

Treatment of the animals

- *Group Tto_1*: These animals underwent treatment during the fibrosis process. Treatment with the peptide started in the fifth week (before exposure to CCl_4 for 5 minutes) and continued up to the end of the eleven weeks of the cirrhosis induction process.
- *Group Tto_2*: These animals underwent treatment after completion of the process of induction of cirrhosis (11 weeks). Treatment started one week after the last inhalation of CCl_4 and continued for 21 days.

Before starting the treatment and on its completion, blood was taken from all the animals that had been treated with the peptide. The peptide was administered by subcutaneous injection in the abdominal zone at a dose of 70 µg/animal in 500 µl of physiological saline.

Sacrifice of the animals and dissection of the liver

On completion of treatment of the animals with the peptide, both in the model with rats and in that with mice, the animals were sacrificed by decapitation, after taking blood from them from the retro-orbital plexus with a capillary.

This was followed immediately by dissection of the liver and collection of samples.

The samples were cut and placed in formol as fixing solution, for later histologic examination. Other fragments were placed in cryotubes, which were immersed in liquid nitrogen and then stored at -80°C.

Anatomopathologic evaluation of the liver

Histologic examination was carried out on fragments of liver previously fixed in formol for at least 24 hours, after which they were placed in ethanol (70%).

After dehydrating they were embedded in paraffin blocks. Successive sections 3 µm thick were prepared from the blocks obtained, using a Leitz rotary microtome and steel blades. Prior to staining the sections were deparaffined in xylene (*AnalaR, BDH, Poole, UK*) for 15 minutes, after heating them at 60°C in a stove for 15 minutes, and they were hydrated by successive passes through alcohols of decreasing concentration 100%, 96%, 80% and 70% and finally in water. The following stains were used:

Haematoxylin-eosin.

Masson's trichromic (Locquin M. and Langeron, (1985) in Manual de Microscopía Ed. Labor S.A. Barcelona): Uses a specific dye for collagen proteins (green light).

Sirius Red: A stain specific for collagen.

Confirmation of hepatic fibrosis: image analysis

For image analysis of the samples obtained, a light microscope was used (*Olympus BH-2, Tokyo, Japan*) connected to a video camera (*Sony DXP-950P, Sony Co., Tokyo, Japan*), with which the various fields of each preparation were photographed. Six fields were taken at random from each preparation stained with Sirius Red. The various images captured were analysed by means of a computer program (*Visilog 4.1.5, Noesis, Orsay, France*) which calculates the area of fibrosis and the total area of the preparation. From these data, a fibrosis index (area of fibrosis/total area) was calculated for each field. To be able to use this program it was necessary to modify image acquisition by using polarized light filters (*Olympus U-POT, Tokyo, Japan*) and green light filters (*Olympus IF550, Tokyo, Japan*) which made it possible to automate the process of sample analysis.

Detection of collagen in 14 µm sections of paraffin-treated tissue

The 14 µm sections that were used for this technique were obtained in the same way as the 3 µm sections mentioned previously. These sections were subjected to a process of deparaffination for 12 hours in xylene. Once the paraffin had been eliminated, the samples were hydrated by passing them through different grades of alcohol 96%, 80%, 50%, completing the process in distilled water.

Once hydrated, they were subjected to a process of prestaining in a solution of 160 mg of *Fast Green FCF* (*Fluka Chemika-BioChemika, Buchs, Switzerland*) in 160 ml of saturated picric acid (*Merck, Darmstadt, Germany*) for 15 minutes in darkness. The samples were

washed by immersion in water until they no longer coloured the wash water. Once the surplus dye was removed, the samples were stained for 30 minutes in darkness in a solution of 160 mg of *Direct Red 80* (*Fluka Chemika-BioChemika Buchs, Switzerland*) and 64 mg of *Fast Green*, both dyes in 160 ml of saturated picric acid. They were washed again until the surplus dye was removed, and then the samples were removed from the slides by scraping the sample off with a small spatula. The sections removed in this way were placed in separate tubes containing 3 ml of a solution of NaOH 0.1 N (*Quimón, Montplet&Esteban S.A., Barcelona, Spain*) and methanol (1:1). Aliquots were taken from the various tubes for reading in the spectrophotometer (*Lambda 2 UV/VIS spectrophotometer, Perkin-Elmer, Norwalk, USA*) at wavelengths of 540 nm and 630 nm using as blank an aliquot of the solution of NaOH 0.1 N and methanol (López de León A. and Rojkind (1985) Histochem. Cytochem. 33:737-743; Gaudio E. et al. (1993) Int. J. Exp. Path. 74:463-469).

In accordance with the works of Gaudio E. et al. (1993) Int. J. Exp. Path. 74:463-469), the following formulae were used for finding the quantities of collagen and total protein:

$$\text{mg collagen} = \frac{\text{absorbance at 540 nm - absorbance at 630 nm}}{37}$$

$$\text{mg collagen/mg total protein} = \frac{\text{mg collagen}}{\text{mg collagen + mg non-collagen proteins}}$$

$$\text{Non-collagen proteins} = \frac{\text{absorbance at 630 nm}}{3}$$

Statistical analysis of the results

The data obtained in the experiments *in vivo* were subjected to statistical analysis. Normality of the

quantitative variables was verified by the Shapiro-Wilks test.

As the data had not been adjusted to a normal distribution, non-parametric statistical analysis was undertaken. Comparison between groups was effected by means of Kruskal-Wallis H followed by comparison of Mann-Whitney U. The data were presented graphically by means of boxes, with representation of the median of the data (thick line inside each box), together with the interquartile range (height of the box), whereas the "whiskers" of each box represent the highest and lowest observations within a given interquartile range.

The association between variables was investigated using Fisher's exact test. Logistic regression was employed for investigating the independence of association of these variables.

A value of P equal to or less than 0.05 was regarded as significant.

All the statistical analyses were accomplished using the program SPSS for Windows V 6.1.3.

INHIBITION *IN VITRO* OF THE ACTIVITY OF TGFβ1

Test of inhibition of cell growth of the MV-1-Lu line

TGFβ1 is a cytokine that is able to inhibit the growth *in vitro* of the MV-1-Lu cell line (Grubeck-Loebenstein B. et al. (1989) J. Clin. Invest. 83:764-770; Brennan FM et al. (1990) Clin. Exp. Immunol. 81:278-285), therefore this line was used for testing the blocking effect of peptides on TGFβ1. After different combinations of media, cells and thymidine, we studied the effect of different concentrations of TGFβ1 on incorporation of [methyl-^{3}H]thymidine by MV-1-Lu cells in culture, for determining the most

suitable conditions for the test. These conditions are shown in Fig. 3.

Once both the optimum concentration of MV-1-Lu cells (5000 cells/well) and the lowest concentration of TGFβ1 capable of producing inhibition of about 90% (200 pg/ml, Fig. 18) had been determined, the inhibitory effect of the synthetic peptides at a concentration of 200 µg/ml was tested.

Inhibition in vitro of the activity of TGFβ1 by synthetic peptides

The synthetic peptides that are potentially inhibitors of TGFβ1 activity, selected as indicated above in the section: selection of the peptides to be synthesized (both those derived from proteins that bind to TGFβ1 and TGFβ1 itself) were tested using the MV-1-Lu cell line. The peptides were dissolved in buffered RPMI medium, free from foetal calf serum, and the following procedure was used:

The peptides belonging to the sequence of the receptor, or complementary to the peaks of hydrophilicity of TGFβ1, were incubated for 30 minutes in the presence of this cytokine and were then combined with the cell culture. The peptides derived from the sequence of TGFβ1 were added to the cell culture prior to addition of TGFβ1, since they interact with the receptors of the cell surface. These incubations were effected in 100 µl of the same medium as was used for adding the cells. The active peptides permitted cell growth to a greater or lesser degree depending on its ability to inhibit TGFβ1.

Inhibition of TGFβ1 by means of peptides derived from TGFβ1

In a first stage, overlapping peptides derived from TGFβ1 were synthesized. These peptides (Table 2) were synthesized in the hope that some of them could bind to the cell receptors, thus preventing the binding of natural TGFβ1 to these receptors.

Table 2. Peptides derived from TGFβ1. The number of the peptide is shown, together with its position in the complete sequence, as well as its amino acid sequence. For convenience of synthesis, all the peptides were synthesized with an alanine added at the C-terminal end which is not shown in the table.

Peptide	Sequence
$P1_{(280-293)}$	AlaLeuAspThrAsnTyrCysPheSerSerThrGluLysAsn
$P2_{(284-297)}$	AsnTyrCysSerSerThrGluLysAsnCysCysValArg
$P3_{(288-301)}$	SerSerThrGluLysAsnCysCysValArgGlnLeuTyrIle
$P4_{(294-307)}$	CysCysValArgGlnLeuTyrIleAspPheArgLysAspLeu
$P5_{(298-311)}$	GlnLeuTyrIleAspPheArgLysAspLeuGlyTrpLysTrp
$P6_{(302-315)}$	AspPheArgLysAspLeuGlyTrpLysTrpIleHisGluPro
$P7_{(306-319)}$	AspLeuGlyTrpLysTrpIleHisGluProLysGlyTyrHis
$P8_{(308-321)}$	GlyTrpLysTrpIleHisGluProLysGlyTyrHisAlaAsn
$P9_{(312-325)}$	IleHisGluProLysGlyTyrHisAlaAsnPheCysLeuGly
$P10_{(316-329)}$	LysGlyTyrHisAlaAsnPheCysLeuGlyProCysProTyr
$P11_{(319-333)}$	HisAlaAsnPheCysLeuGlyProCysProTyrIleTrpSerL u
$P12_{(322-335)}$	PheCysLeuGlyProCysProTyrIleTrpSerLeuAspThr
$P13_{(326-339)}$	ProCysProTyrIleTrpSerLeuAspThrGlnTyrSerLys
$P14_{(330-343)}$	IleTrpSerLeuAspThrGlnTyrSerLysValLeuAlaLeu
$P15_{(335-349)}$	ThrGlnTyrSerLysValLeuAlaLeuTyrAsnGlnHisAsnPro
$P16_{(336-349)}$	GlnTyrSerLysValLeuAlaLeuTyrAsnGlnHisAsnPro
$P17_{(340-353)}$	ValLeuAlaLeuTyrAsnGlnHisAsnProGlyAlaSerAla
$P18_{(343-358)}$	LeuTyrAsnGlnHisAsnProGlyAlaSerAlaAlaProCysCys
$P19_{(344-358)}$	TyrAsnGlnHisAsnProGlyAlaSerAlaAlaProCysCys
$P20_{(348-360)}$	AsnProGlyAlaSerAlaAlaProCysCysValProGln
$P21_{(350-363)}$	GlyAlaSerAlaAlaProCysCysValProGlnAlaLeuGlu
$P22_{(354-367)}$	AlaProCysCysValProGlnAlaLeuGluProLeuProIle
$P23_{(358-371)}$	ValProGlnAlaLeuGluProLeuProIleValTyrTyrVal
$P24_{(364-377)}$	ProLeuProIleValTyrTyrValGlyArgLysProLysVal
$P25_{(368-381)}$	ValTyrTyrValGlyArgLysProLysValGluGlnLeuSer
$P26_{(372-385)}$	GlyArgLysProLysValGluGlnLeuSerAsnMetIleVal
$P27_{(378-391)}$	GluGlnLeuSerAsnMetIleValArgSerCysLysCysSer

Fig. 4 shows the inhibitory effect of the peptides in Table 6 on the activity of TGFβ1. Since TGFβ1 inhibits growth of the MV-1-Lu cells, inhibition of this cytokine by the peptides leads to re-establishment of growth of the MV-1-Lu cells.

As can be seen from Fig. 4, the peptide P12, derived from the sequence of TGFβ1, is the one that exhibits greater inhibitory activity of TGFβ1. For more detailed investigation of the inhibitory effect of peptide P12, an investigation was conducted into the effect of the concentration of the peptide on inhibition of the cytokine, which is described below.

Dose-response test of the inhibition of TGFβ1 by the peptide P12

The effect of the concentration of peptide P12 on inhibition of the activity of TGFβ1 was investigated. As this peptide was not readily soluble in the test medium, stock solutions or suspensions were prepared with a nominal concentration of peptide (that which would have been achieved if the peptide had dissolved completely) and aliquots were taken from these, and were filtered or even were used directly for the inhibition tests.

Fig. 5 examines the inhibitory effect of nominal concentrations of peptide, before and after filtration. It can be seen that peptide P12, with and without filtration, has practically the same activity.

Once the results had been obtained with peptide P12, it was decided to lengthen the peptide, both in the N-terminal and the C-terminal direction, and to investigate the effect on its activity. In addition, changes were made to its sequence to improve its solubility and study the importance of the two cysteines in its sequence on the inhibitory activity of TGFβ1. The peptides synthesized are stated in Table 3.

Table 3. Peptides derived from modification of peptide P12.

Peptide	Sequence
$P12_{(322-335)}$	PheCysLeuGlyProCysProTyrIleTrpSerLeuAspThr
$P28_{(322-344)}$	PheCysLeuGlyProCysProTyrIleTrpSerLeuAspThrGlnLysVal LeuAlaLeuTyr
$P29_{(313-335)}$	HisGluProLysGlyTyrHisAlaAsnPheCysLeuGlyProCysProTyr IleTrpSerLeuAspThr
P30	PheSerLeuGlyProCysProTyrIleTrpSerLeuAspThr
P31	PheCysLeuGlyProSerProTyrIleTrpSerLeuAspThr
P32	PheSerLeuGlyProSerProTyrIleTrpSerLeuAspThr
P33	PheCysLeuGlyProCysProTyrIleTrpSerAspAspAsp
P34	AspAspAspGlyProCysProTyrIleTrpSerLeuAspThr
P35	AspAspAspGlyProCysProTyrIleTrpSerAspAspAsp
P36	GlyProCysProTyrIleTrpSerAspAspAsp
P37	AspAspAspGlyProCysProTyrIleTrpSer
P38	AspGlyProCysProTyrIleTrpSerAsp

Fig. 6 shows the results of inhibition of TGFβ1 by the peptides in Table 3.

It can be seen from Fig. 6 that peptide P29 is active. This peptide includes the previously tested peptide P12 and has 9 extra amino acids towards the N-terminal end (Fig. 4). Investigations conducted by Quian SW et al. (1992) Proc. Natl. Acad. Sci. 89:6290-6294) and by Burmester JK et al. (1993) Proc. Natl. Acad. Sci. 90:8628-8632) using chimeric recombinant proteins identified a region of TGFβ1 that is necessary for the activity of this cytokine (amino acids 40 to 82 in the sequence of mature TGFβ1). It was speculated that peptide P29 (amino acids 34 to 56 in the sequence of mature TGFβ1), extending over a larger region than peptide P12 (amino acids 43 to 56), might acquire a three-dimensional structure more like the structure of the TGFβ1 in circulation. For this reason, peptide P29 was used for tests of binding to the cell receptors, based on affinity labelling.

Tests of inhibition of the binding of TGFβ1 to its receptors by peptide P29 (affinity labelling)

Peptide P29, derived from the sequence of TGFβ1, was used in affinity labelling tests for verifying its capacity for inhibition of the binding of TGFβ1 to its cell receptors (Material and Methods).

Owing to the different activity of the batches of ^{125}I-TGFβ1 employed, the concentrations of peptide used in the tests were adjusted according to the concentration of the ^{125}I-TGFβ1 batch used in each case. The results of these tests are shown in Figs. 7 and 8.

Further tests were carried out to find the minimum concentration required for blocking the binding of ^{125}I-TGFβ1 to the cell receptors.

Inhibition of TGFβ1 by peptides derived from the sequence of the type III receptor of the rat

With the aim of finding new peptides that are inhibitors of the activity of TGFβ1, peptides derived from the type III receptor of the rat were synthesized. Some peptides were chosen on the basis of regions of their sequence that were predicted as complementary to blocks of amino acids of the sequence of TGFβ1. It was hoped that these peptides would be capable of binding to free TGFβ1, sequestering it and preventing its binding to the cell receptors.

Other peptides were synthesized by overlapping 10 amino acids and covering part of the extracellular region of the type III receptor (amino acids 45 to 410). It has been described that a soluble type III receptor exists that corresponds to the extracellular region of the receptor, this region is cut from the membrane and acts as a sequestrator of the TGFβ1 in

circulation (López Casillas F. et al. (1991) Cell 67:785-795). Later studies described two possible regions of binding to TGFβ1, one of which is located at the N-terminal end of the receptor (López-Casillas et al. (1994) J. Cell Biol. 124:557-568) and the other is located in the region closest to the membrane, towards the C-terminal end (Fukushima D. et al. (1993) J. Biol. Chem. 268:22710-22715; Pepin MC et al. (1995) FEBS Lett 377:368-372). For these reasons peptides of the extracellular region of this receptor were synthesized, on the supposition that these peptides might be capable of sequestering the circulating TGFβ1.

The peptides synthesized are shown in Table 4.

Table 4. Peptides derived from the type III receptor of the rat. The number of the peptide and its sequence are shown. P39 to P65 are peptides predicted as complementary to TGFβ1 and P66 to P138 are overlapping peptides covering the extracellular region of the receptor. For convenience of synthesis, all the peptides were synthesized with an alanine added at the C-terminal end which is not shown in the table.

Peptide	Sequence
P39 (91-102)	AsnProIleAlaSerValHisThrHisHisLysPro
P40 (104-115)	ValPheLeuLeuAsnSerProGlnProLeuValTrp
P41 (109-120)	SerProGlnProLeuValTrpHisLeuLysThrGlu
P42 (110-121)	ProGlnProLeuValTrpHisLeuLysThrGluArg
P43 (333-344)	TrpAlaLeuAspAsnGlyTyrArgProValThrSer
P44 (428-439)	ProIleValProSerValGlnLeuLeuProAspHis
P45 (555-566)	GlyAspGluGlyGluThrAlaProLeuSerArgAla
P46 (563-574)	LeuSerArgAlaGlyValValValPheAsnCysSer
P47 (603-614)	LeuPheLeuValProSerProGlyValPheSerVal
P48 (605-616)	LeuValProSerProGlyValPheSerValAlaGlu
P49 (707-718)	GluLeuThrLeuCysSerArgLysLysGlySerLeu
P50 (712-723)	SerArgLysLysGlySerLeuLysLeuProArgCys
P51 (717-728)	SerLeuLysLeuProArgCysValThrProAspAsp
P52 (722-733)	ArgCysValThrProAspAspAlaCysThrSerLeu
P53 (727-738)	AspAspAlaCysThrSerLeuAspAlaThrMetIle
P54 (731-742)	ThrSerLeuAspAlaThrMetIleTrpThrMetMet
P55 (732-743)	SerLeuAspAlaThrMetIleTrpThrMetMetGln
P56 (737-748)	MetIleTrpThrMetMetGlnAsnLysLysThrPhe
P57 (742-752)	MetGlnAsnLysLysThrPheThrLysProLeuAla
P58 (747-758)	ThrPheThrLysProLeuAlaValValLeuGlnVal
P59 (761-775)	LysGluAsnValProSerThrLysAspSerSerProIleProPro
P60 (766-780)	SerThrLysAspSerSerProIleProProProProProGlnIle
P61 (771-785)	SerProIleProProProProProGlnIlePheHisGlyLeuAsp
P62 (776-790)	ProProProGlnIlePheHisGlyLeuAspThrLeuThrValMet
P63 (781-795)	PheHisGlyLeuAspThrLeuThrValMetGlyIleAlaPheAla
P64 (786-800)	ThrLeuThrValMetGlyIleAlaPheAlaAlaPheValIleGly
P65 (797-809)	LeuLeuThrGlyAlaLeuTrpTyrIleTyrSerHis
P66 (45-59)	LeuMetGluSerPheThrValLeuSerGlyCysAlaSerArgGly
P67 (50-64)	ThrValLeuSerGlyCysAlaSerArgGlyThrThrGlyLeuPro
P68 (55-69)	CysAlaSerArgGlyThrThrGlyLeuProArgGluValHisVal
P69 (60-74)	ThrThrGlyLeuProArgGluValHisValLeuAsnLeuArgSer
P70 (65-79)	ArgGluValHisValLeuAsnLeuArgSerThrAspGlnGlyPro
P71 (70-84)	LeuAsnLeuArgSerThrAspGlnGlyProGlyGlnArgGlnArg
P72 (75-89)	ThrAspGlnGlyProGlyGlnArgGlnArgGluValThrLeuHis
P73 (80-94)	GlyGlnArgGlnArgGluValThrLeuHisLeuAsnProIleAla

P74 (85-99)	GluValThrLeuHisLeuAsnProIleAlaSerValHisThrHis
P75 (90-104)	LeuAsnProIleAlaSerValHisThrHisHisLysProIleVal
P76 (95-109)	SerValHisThrHisHisLysProIleValPheLeuLeuAsnSer
P77 (100-114)	HisLysProIleValPheLeuLeuAsnSerProGlnProLeuVal
P78 (105-119)	PheLeuLeuAsnSerProGlnProLeuValTrpHisLeuLysThr
P79 (110-124)	ProGlnProLeuValTrpHisLeuLysThrGluArgLeuAlaAla
P80 (115-129)	TrpHisLeuLysThrGluArgLeuAlaAlaGlyValProArgLeu
P81 (120-134)	ArgLeuAlaAlaGlyValProArgLeuPheLeuValSerGluGly
P82 (125-139)	GlyValProArgLeuPheLeuValSerGluGlySerValValGln
P83 (130-144)	PheLeuValSerGluGlySerValValGlnPheProSerGlyAsn
P84 (135-149)	GlySerValValGlnPheProSerGlyAsnPheSerLeuThrAla
P85 (140-154)	PheProSerGlyAsnPheSerLeuThrAlaGluThrGluGluArg
P86 (145-159)	PheSerLeuThrAlaGluThrGluGluArgAsnPheProGlnGlu
P87 (150-164)	GluThrGluGluArgAsnPheProGlnGluAsnGluHisLeuVal
P88 (155-169)	AsnPheProGlnGluAsnGluHisLeuValArgTrpAlaGlnLys
P89 (160-174)	AsnGluHisLeuValArgTrpAlaGlnLysGluTyrGlyAlaVal
P90 (165-179)	ArgTrpAlaGlnLysGluTyrGlyAlaValThrSerPheThrGlu
P91 (170-184)	GluTyrGlyAlaValThrSerPheThrGluLeuLysIleAlaArg
P92 (175-189)	ThrSerPheThrGluLeuLysIleAlaArgAsnIleTyrIleLys
P93 (180-194)	LeuLysIleAlaArgAsnIleTyrIleLysValGlyGluAspGln
P94 (185-199)	AsnIleTyrIleLysValGlyGluAspGlnValPheProProThr
P95 (190-201)	ValGlyGluAspGlnValPheProProThrCysAsnIleGlyLys
P96 (195-209)	ValPheProProThrCysAsnIleGlyLysAsnPheLeuSerLeu
P97 (200-214)	CysAsnIleGlyLysAsnPheLeuSerLeuAsnTyrLeuAlaGlu
P98 (205-219)	AsnPheLeuSerLeuAsnTyrLeuAlaGluTyrLeuGlnProLys
P99 (210-224)	AsnTyrLeuAlaGluTyrLeuGlnProLysAlaAlaGluGlyCys
P100 (215-229)	TyrLeuGlnProLysAlaAlaGluGlyCysValLeuProSerGln
P101 (220-234)	AlaAlaGluGlyCysValLeuProSerGlnProHisGluLysGlu
P102 (225-239)	ValLeuProSerGlnProHisGluLysGluValHisIleIleGlu
P103 (230-244)	ProHisGluLysGluValHisIleIleGluLeuIleThrProSer
P104 (235-249)	ValHisIleIleGluLeuIleThrProSerSerAsnProTyrSer
P105 (240-254)	LeuIleThrProSerSerAsnProTyrSerAlaPheGlnValAsp
P110 (265-279)	AspProGluValValLysAsnLeuValLeuIleLeuLysCysLys
P111 (270-284)	LysAsnLeuValLeuIleLeuLysCysLysLysSerValAsnTrp
P112 (275-289)	IleLeuLysCysLysLysSerValAsnTrpValIleLysSerPhe
P113 (280-294)	LysSerValAsnTrpValIleLysSerPheAspValLysGlyAsn
P114 (285-299)	ValIleLysSerPheAspValLysGlyAsnLeuLysValIleAla
P115 (290-304)	AspValLysGlyAsnLeuLysValIleAlaProAsnSerIleGly

P106(245-259) SerAsnProTyrSerAlaPheGlnValAspIleIleValAspIle
P107(250-264) AlaPheGlnValAspIleIleValAspIleArgProAlaGlnGlu
P108(255-269) IleIleValAspIleArgProAlaGlnGluAspProGluValVal
P109(260-274) ArgProAlaGlnGluAspProGluValValLysAsnLeuValLeu
P116(295-309) LeuLysValIleAlaProAsnSerIleGlyPheGlyLysGluSer
P117(300-314) ProAsnSerIleGlyPheGlyLysGluSerGluArgSerMetThr
P118(305-319) PheGlyLysGluSerGluArgSerMetThrMetThrLysLeuVal
P119(310-324) GluArgSerMetThrMetThrLysLeuValArgAspAspIlePro
P120(315-329) MetThrLysLeuValArgAspAspIleProSerThrGlnGluAsn
P121(320-334) ArgAspAspIleProSerThrGlnGluAsnLeuMetLysTrpAla
P122(325-339) SerThrGlnGluAsnLeuMetLysTrpAlaLeuAspAsnGlyTyr
P123(330-344) LeuMetLysTrpAlaLeuAspAsnGlyTyrArgProValThrS r
P124(335-349) LeuAspAsnGlyTyrArgProValThrSerTyrThrMetAlaPro
P125(340-354) ArgProValThrSerTyrThrMetAlaProValAlaAsnArgPhe
P126(345-359) TyrThrMetAlaProValAlaAsnArgPheHisLeuArgLeuGlu
P127(350-364) ValAlaAsnArgPheHisLeuArgLeuGluAsnAsnGluGluM t
P128(355-369) HisLeuArgLeuGluAsnAsnGluGluMetArgAspGluGluVal
P129(360-374) AsnAsnGluGluMetArgAspGluGluValHisThrIleProPr
P130(365-379) ArgAspGluGluValHisThrIleProProGluLeuArgIleLeu
P131(370-384) HisThrIleProProGluLeuArgIleLeuLeuAspProAspHis
P132(375-389) GluLeuArgIleLeuLeuAspProAspHisProProAlaLeuAsp
P133(380-394) LeuAspProAspHisProProAlaLeuAspAsnProLeuPhePro
P134(385-399) ProProAlaLeuAspAsnProLeuPheProGlyGluGlySerPro
P135(390-404) AsnProLeuPheProGlyGluGlySerProAsnGlyGlyLeuPro
P136(395-409) GlyGluGlySerProAsnGlyGlyLeuProPheProPheProAsp
P137(400-414) AsnGlyGlyLeuProPheProPheProAspIleProArgArgGly
P138(405-419) PheProPheProAspIleProArgArgGlyTrpLysGluGlyGlu

C3

The peptides in Table 4 were tested for their capacity to block TGFβ1 in the model of inhibition of the MV-1-Lu cell line. Since TGFβ1 is able to inhibit the growth of this line, inhibition of TGFβ1 by the peptides would be able to re-establish cell growth. These tests are shown in Figs. 9 to 12.

As can be seen in Figs. 9 to 12, there are various peptides that are able to inhibit the growth of the MV-1-Lu cell line to a greater or lesser degree, but only peptide P54 is capable of inhibiting the activity of TGFβ1 almost completely. With the aim of conducting a more thorough investigation of this peptide, tests were carried out using different concentrations of peptide against a fixed concentration of TGFβ1 of 200 pg/ml.

Dose-response test of the inhibition of TGFβ1 by peptide P54

The effect of the concentration of peptide P54 on inhibition of the activity of TGFβ1 was investigated. In view of the low solubility of this peptide, stock solutions with nominal concentration of peptide were prepared, as was done in the case of peptide P12, and aliquots were taken from them, and filtered, or even used directly for the inhibition tests.

Fig. 13 examines the inhibitory effect of nominal concentrations of peptide, before and after filtration. It can be seen that there is no measurable inhibitory activity in the filtrate of peptide P54.

Having verified the capacity of peptide P54 to inhibit the activity of TGFβ1 in a manner that depends on the dose used, we proceeded to synthesize new peptides, taking as a basis the sequence of P54, with the aim of trying to improve the solubility and hence its activity at lower doses. Two peptides derived from

the human type III receptor were also synthesized. One of these peptides (P144) is equivalent to peptide P54. The other peptide (P145) is similar to peptide P106 of the type III receptor of the rat, which had also demonstrated activity. These new peptides are shown in Table 5.

Table 5. Peptides derived from modification of peptide P54 (peptides P139 to P143) and of the human type III receptor (peptides P144 and P145).

Peptide	Sequence	Derivation
$P54_{(731-742)}$	ThrSerLeuAspAlaThrMetIleTrpThrMetMet	Rat type III receptor
P139	ThrSerLeuAspAlaThrMetIleTrpAspAspAsp	
P140	AspAspAspAlaThrMetIleTrpThrMetMet	
P141	AspAlaThrMetIleTrpAsp	
P142	ThrSerLeuMetIleTrpThrMetMet	
P143	ThrSerLeuAspAlaThrThrMetMet	
$P144_{(729-742)}$	ThrSerLeuAspAlaSerIleIleTrpAlaMetMet GlnAsn	Human type III receptor
$P145_{(241-254)}$	SerAsnProTyrSerAlaPheGlnValAspIleThr IleAsp	Human type III receptor

The test of activity of the peptides in Table 5 is shown in Fig. 14.

Dose-response test of inhibition of TGFβ1 by peptide P144

A dose-response test was carried out with peptide P144 derived from the sequence of the human type III receptor, with the aim of testing whether its activity was dependent on the concentration (Fig. 15). It can be seen that the activity of the peptide decreases with the decrease in the concentration of peptide used in the tests.

Tests of inhibition of the binding of TGFβ1 to its receptors by peptide P144 (affinity labelling)

Peptide P144 derived from the sequence of the human type III receptor was used in affinity labelling tests for verifying its ability to inhibit the binding of TGFβ1 to its cell receptors (Material and Methods).

Owing to the different activity of the batches of ^{125}I-TGFβ1 employed, the concentrations of peptide used in the tests were adjusted according to the concentration of the ^{125}I-TGFβ1 batch used in each case. The results of these tests are shown in Fig. 15.

After verifying inhibition of the binding of TGFβ1 to its cell receptors by peptide P144, a new test was conducted with the aim of titrating peptide P144. It was observed that the peptide lost its activity at a concentration of $2x10^5$ times the molar concentration of ^{125}I-TGFβ1.

Inhibition of TGFβ1 by peptides derived from other proteins with ability to bind to TGFβ1 and predicted as complementary to TGFβ1

The peptides in Table 6, derived from proteins capable of binding to TGFβ1, were synthesized in this series.

Table 6. Peptides derived from various proteins capable of binding to TGFβ1 (type II receptor P146, fetuin P147 to P149, endoglin P150 to P154 and α2-macroglobulin P155 to P179). The number of the peptide is shown, together with its position in the complete sequence, its amino acid sequence, and its origin. For convenience of synthesis, all the peptides were

synthesized with an alanine added at the C-terminal end which is not shown in the table.

Peptide	Sequence	Origin
P146(84-101)	CysValAlaValTrpArgLysAsnAspGluAsnIleThr LeuGluThrValCys	Type II receptor
P147(114-132)	CysAspPheGlnLeuLeuLysLeuAspGlyLysPheSer ValValTyrAlaLysCys	Fetuin
P148(114-132)	CysAspPheHisIleLeuLysGlnAspGlyGlnPheArg ValCysHisAlaGlnCys	Fetuin
P149(114-132)	CysAspIleHisValLeuLysGlnAspGlyPheSerVal LeuPheThrLysCysAsp	Fetuin
P150(247-261)	GluAlaValLeuIleLeuGlnGlyProProTyrValSer TrpLeu	Endoglin
P151(289-303)	ValAsnLeuProAspThrArgGlnGlyLeuLeuGluGlu AlaArg	Endoglin
P152(445-459)	LeuAspSerLeuSerPheGlnLeuGlyLeuTyrLeuSer ProHis	Endoglin
P153(481-495)	ProSerIleProGluLeuMetThrGlnLeuAspSerCys GlnLeu	Endoglin
P154(479-493)	MetSerProSerIleProGluLeuMetThrGlnLeuAsp SerCys	Endoglin
P155(13-24)	LeuLeuLeuLeuValLeuLeuProThrAspAlaSer	α-2-Macroglobulin
P156(30-31)	ProThrAspAlaSerValSerGlyLysProGlnTyr	α-2-Macroglobulin
P157(44-55)	ThrGluLysGlyCysValLeuLeuSerTyrLeuAsn	α-2-Macroglobulin
P158(166-177)	TyrIleGlnAspProLysGlyAsnArgIleAlaGln	α-2-Macroglobulin
P158(166-177)	TyrIleGlnAspProLysGlyAsnArgIleAlaGln	α-2-Macroglobulin
P159(193-203)	PheProLeuSerSerGluProPheGlnGlySerTyr	α-2-Macroglobulin
P160(247-258)	AsnValSerValCysGlyLeuTyrThrTyrGlyLys	α-2-Macroglobulin
P161(248-259)	ValSerValCysGlyLeuTyrThrTyrGlyLysPro	α-2-Macroglobulin
P162(250-261)	ValCysGlyLeuTyrThrTyrGlyLysProValPro	α-2-Macroglobulin
P163(267-278)	SerIleCysArgLysTyrSerAspAlaSerAspCys	α-2-Macroglobulin
P164(469-480)	ProCysGlyHisThrGlnThrValGlnAlaHisTyr	α-2-Macroglobulin
P165(554-565)	AspSerAlaLysTyrAspValGluAsnCysLeuAla	α-2-Macroglobulin
P167(790-801)	GlnProPhePheValGluLeuThrMetProTyrSer	α-2-Macroglobulin
P168(827-838)	GlnLeuGluAlaSerProAlaPheLeuAlaValPro	α-2-Macroglobulin
P169(835-836)	SerValGlnLeuGluAlaSerProAlaPheLeuAla	α-2-Macroglobulin
P170(876-887)	AlaLeuGluSerGlnGluLeuCysGlyThrGluVal	α-2-Macroglobulin
P171(1001-1012	LysSerLysIleGlyTyrLeuAsnThrGlyTyr	α-2-Macroglobulin

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P172 (1005-1016)	IleGlyTyrL uAsnThrGlyTyrGlnArgGlnLeu	α-2-Macroglobulin
P173 (1062-1073)	LysArgLysGluValLeuLysSerLeuAsnGluGlu	α-2-Macroglobulin
P174 (1193-1204)	ValGlyHisPheTyrGluProGlnAlaProSerAla	α-2-Macroglobulin
P175 (1209-1220)	ThrSerTyrValLeuLeuAlaTyrLeuThrGlnAla	α-2-Macroglobulin
P176 (1211-1222)	TyrValLeuLeuAlaTyrLeuThrAlaGlnProAla	α-2-Macroglobulin
P177 (1256-1267)	ValAlaLeuHisAlaLeuSerLysTyrGlyAlaAla	α-2-Macroglobulin
P178 (1232-1243)	TyrGlyArgAsnGlnGlyAsnThrTrpLeuThrAla	α-2-Macroglobulin
P179 (1234-1245)	ArgAsnGlnGlyAsnThrTrpLeuThrAlaPheVal	α-2-Macroglobulin

Figs. 17 and 18 show the inhibitory activity of the peptides derived from Table 10.

As can be seen in Figs. 17 and 18, only peptide P150 showed activity greater than 50%. However, peptides P146 and P149, which had been described as active by Demetriou M et al. (1996) J. Biol. Chem. 271:12755-12761, were not found to be active under the conditions employed for this test.

Measurement by flow cytometry of the inhibitory effect of synthetic peptides on the binding of TGFβ1 to its cell receptors

Peptides derived from previous syntheses, both those that were synthesized from the sequence of TGFβ1 and those from the type III receptor, were used for measuring, by flow cytometry, their capacity to inhibit the binding of TGFβ1 to the cell receptors. In these tests the cells are incubated with the peptide before adding TGFβ1-biotin, which will be detected using avidin-FITC (Material and Methods). Then the fluorescence emitted by the avidin-FITC is measured: this will be directly proportional to the quantity of TGFβ1 bound to the cells and inversely proportional to the activity of the peptide. The results obtained with the most relevant peptides are shown in Fig. 19 and Table 7.

Table 7. Comparison of the inhibitory activity of TGFβ1, of some peptides, measured by bioassay of inhibition of growth of the MV-1-Lu[1] cells (peptide concentration 200 µg/ml) with inhibition of the binding of TGFβ1 to its cell receptors measured using flow cytometry[2] (peptide concentration 420 µg/ml).

Peptides	Bioassay (% inhibition)[1]	Cytometry % inhibition)[2]	Sequence
P29	77,6	92,34	HisGluProLysGlyTyrHisAlaAsnPheCysLeuGlyProCysProTyrIleTrpSerLeuAspThr
P11	40	86	HisAlaAsnPheCysLeuGlyProCysProTyrIleTrpSerLeu
P12	96	77	PheCysLeuGlyProCysProTyrIleTrpSerLeuAspThr
P18	18,2	6,5	LeuTyrAsnGlnHisAsnProGlyAlaSerAlaAlaProCysCys
P54	97	82,3	ThrSerLeuAspAlaThrMetIleTrpThrMetMet
P140	-1,7	69,8	AspAspAspAlaThrMetIleTrpThrMetMet
P142	70	72	ThrSerLeuMetIleTrpThrMetMet
P106	40	91	SerAsnProTyrSerAlaPheGlnValAspIleIleValAspIle
P145	21	74,35	SerAsnProTyrSerAlaPheGlnValAspIleThrIleAsp
P144	88	80	ThrSerLeuAspAlaSerIleIleTrpAlaMetMetGlnAsn
P150	64	73	GluAlaValLeuIleLeuGlnGlyProProTyrValSerTrpLeu
P152	45	68,4	LeuAspSerLeuSerPheGlnLeuGlyLeuTyrLeuSerProHis

INHIBITION *IN VIVO* OF THE ACTIVITY OF TGFβ1

Peptide P144 derived from the sequence of the human type III receptor, which had proved active in the bioassays of inhibition of growth of the MV-1-Lu cell line, was used in the tests *in vivo* for studying its inhibitory effect in the induction of experimental cirrhosis with CCl_4, in a rat model.

Model of experimental cirrhosis in Wistar rats

In this model, hepatic cirrhosis is induced by inhalation of carbon tetrachloride, for 11 weeks, twice per week (López Novoa JM et al. (1976) Patología IX:223-240; Camps J. et al. (1987) Gastroenterology 93:498-505) as described in Material and Methods.

Peptide P144 was administered in accordance with two protocols:

1. *Protocol 1*: The peptide was administered on alternate days by the intraperitoneal route during the cirrhosis induction process (11 weeks). Figs. 20 and 21.

2. *Protocol 2*: The peptide was administered on alternate days by the intraperitoneal route for 3 weeks, once cirrhosis was established, i.e. at 12 weeks from the start of induction of cirrhosis. Figs. 22 and 23.

The production of collagen in both protocols was measured by two techniques:

Figs. 36 and 38 show total collagen production measured by staining liver sections (two per animal) with *Fast Green* and *Direct Red*, elution of the colour and reading in a spectrophotometer (Material and Methods) (López de León A. and Rojkind (1985) Histochem. Cytochem. 33:737-743; Gaudio E. et al. (1993) Int. J. Exp. Path. 74:463-469).

Figs. 21 and 23 show collagen production, measured by image analysis of liver sections stained with Sirius Red, using light microscopy (Material and Methods).

As can be seen in Fig. 20, significant differences are observed ($P < 0.05$) between the group of rats treated with peptide P144 (Tto_1) and the control group of cirrhotic rats (Ci_1) on investigating the ratio of collagen to total protein. In Fig. 37, the differences between the group of rats treated with peptide P144 (Tto_1) and the control group of cirrhotic rats (Ci_1) are

also significant ($P < 0.001$) when the area of fibrosis is investigated.

As can be seen in Figs. 22 and 23, which show the results for the rats treated once cirrhosis was established, the differences between the groups of rats treated with peptide P144 (Tto_2) and the cirrhotic rats without treatment (Ci_2) are not significant when using either of the two techniques for measuring fibrosis.

The two techniques employed for measuring collagen were compared using linear regression with the aim of verifying the randomness of selection of the fields for investigation in each preparation and hence the validity of the image analysis, Figs. 24 and 25.

As can be seen from Figs. 24 and 25, there is a correlation between the two techniques with $R > 0.85$ in both cases, which is highly significant ($F \leq 0.001$). This confirms that acquisition of the images for investigation was effected entirely randomly and hence confirms the validity of the data obtained by image analysis.

Figs. 26 and 27 show the images obtained by light microscopy from liver preparations stained with Sirius Red at a magnification of 10X obtained from livers of rats treated during the cirrhosis induction process (Ci_1 and Tto_1).

The images in Fig. 26 were obtained without employing any type of filter.

Fig. 27 shows the images once they had been modified for investigation using special software. These modifications consist of application of two filters, one of polarized light and the other of green light, for the purpose of improving the quality of the images and facilitating automated examination of them.

Figs. 26 and 27 reveal that there are differences between the images obtained from the cirrhotic rats (Ci_1) and those obtained from the rats treated with peptide P144 (Tto_1).

The differences in effectiveness between protocols 1 and 2 might be due to the fact that production of TGFβ1 might be much less once cirrhosis is induced (protocol 2) than during the process of induction of cirrhosis with CCl_4 (protocol 1), and might even be at normal levels, so that the effect of treatment with peptide P144 would be less pronounced in protocol 2 than in protocol 1.

When we compare the groups of untreated cirrhotic rats, at the end of the process of induction of cirrhosis (Ci_1) with the untreated cirrhotic rats at 4 weeks from the end of induction (Ci_2), we find that there are significant differences ($P = 0.016$) between the two groups (Fig. 28), which would indicate that there is partial regression of cirrhosis when the cirrhotizing agent is removed, an observation that has been published by various authors (Szende-B et al. (1992) In Vivo 6:355-361; Columbano A (1996) Carcinogenesis 17:395-400).

These differences in effectiveness between the two protocols might also be due to the protocol itself, since the animals of protocol 2 are only treated for 3 weeks on alternate days, whereas the animals of protocol 1 are treated for a longer period of time (7 weeks, also on alternate days).

The results obtained demonstrate that it is possible to inhibit TGFβ1 both *in vitro* and *in vivo* by means of synthetic peptides derived from different proteins. In future it would be of great interest to try to increase the biological activity of these peptides. This might be accomplished by systematically replacing each of the amino acids of their sequences by the other 19. Once the peptide with greater activity was achieved it would be necessary to prepare mimotopes (McConnell-SJ (1994) Gene 151:115-118; Steward-MW (1995) J. Virol. 69:7668-7673) thereof with the aim of

increasing the average life of the inhibitory agent in the organism.

DESCRIPTION OF THE FIGURES

Fig. 1. Inhibition of binding of TGFβ1 to the MV-1-Lu cells by peptide P144, measured by flow cytometry. A, image obtained on examining the cells incubated with biotinylated TGFβ1 and developed with avidin-FITC. B, image obtained on examining the cells incubated with avidin-FITC without prior addition of TGFβ1. C, image obtained on examining the cells incubated with TGFβ1 previously incubated with peptide P144 at a concentration of 0.42 µg/µl, and developed with avidin-FITC. The fluorescence emitted is shown on the abscissa, while the ordinate shows the number of cells for each value of fluorescence. The fields corresponding to the cells labelled with TGFβ1-biotin and avidin-FITC (M2) and to the unlabelled cells (M1) are also shown.

Fig. 2. Schematic representation of the process of cirrhosis by CCl_4. Black arrows indicate when two weekly doses of CCl_4 were administered to the rats, and black dashed arrows show when there was one weekly dose. The grey arrows indicate administration of peptide P144. A: Healthy controls; B: Healthy controls + P144, B_1: with peptide 70 µg/day; C: Cirrhotic; C_1 with saline; C_2 with peptide 70 µg/day; D: Cirrhotic with CCl_4 + phenobarbital; D_1 plus saline; D_2 plus peptide 70 µg/day.

Fig. 3. Effect of TGFβ1 on growth of MV-1-Lu cells. The cells are cultivated at a density of 5000 cells/well at

the concentrations of TGFβ1 indicated, pg/ml. Abscissa: TGFβ1 concentration (pg/ml); Ordinate: c.p.m.

Fig. 4. Percentage inhibition of TGFβ1 (200 pg/ml) by peptides from TGFβ1. All the peptides were tested at a concentration of 200 µg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 5. Percentage inhibition of the activity of TGFβ1 (200 pg/ml) in the presence of various nominal concentrations of peptide P12, filtered (◆) and unfiltered (•).

Fig. 6. Percentage inhibition of TGFβ1 (200 pg/ml) by peptides from TGFβ1. All the peptides were tested at a concentration of 200 µg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 7. Autoradiograph of an affinity labelling test of the receptors of TGFβ1. Lane C1: effect of incubation of the cells with a concentration of 0.16 µM of ^{125}I-TGFβ1 which corresponds to an activity of 0.3 µCi (positive control). Lane C2: effect of preincubation of the cells with a concentration of non-radioactive TGFβ1 10 times greater than that of ^{125}I-TGFβ1 (negative control). Lane C3: preincubation was effected with peptide P29 at a concentration 10^6 times greater than the molar concentration of ^{125}I-TGFβ1. It can be seen that there is inhibition of the binding of ^{125}I-TGFβ1 to the type I, II and III cell receptors both by peptide P29 and by non-radioactive TGFβ1.

Fig. 8. Autoradiograph of an affinity labelling test of the receptors of TGFβ1. Lanes C1 to C6: effect of preincubation of the MV-1-Lu cells, with different concentrations of peptide P29 (10^6, $8x10^5$, $6x10^5$, $4x10^5$, $2x10^5$ and 10^5 times the molar concentration of ^{125}I-TGFβ1 respectively), prior to addition of ^{125}I-TGFβ1. Lane C7: effect of preincubation of the MV-1-Lu cells with unlabelled TGFβ1 (10^2 times the molar concentration of ^{125}I-TGFβ1) prior to addition of ^{125}I-TGFβ1 (negative control). Lane C8: effect of incubation of the MV-1-Lu cells with a concentration of 0.42 µM of ^{125}I-TGFβ1, corresponding to an activity of 0.4 µCi, without prior preincubation (positive control).

Fig. 9. Percentage inhibition of TGFβ1 (200 pg/ml) by receptor peptides predicted as complementary to regions of TGFβ1. All the peptides were tested at a concentration of 200 µg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 10. Percentage inhibition of TGFβ1 (200 pg/ml) by overlapping peptides derived from the extracellular region of the type III receptor. All the peptides were tested at a concentration of 200 µg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 11. Percentage inhibition of TGFβ1 (200 pg/ml) by overlapping peptides derived from the extracellular region of the type III receptor. All the peptides were tested at a concentration of 200 µg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 12. Percentage inhibition of TGFβ1 (200 pg/ml) by overlapping peptides derived from the extracellular region of the type III receptor. All the peptides were tested at a concentration of 200 μg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 13. Percentage inhibition of the activity of TGFβ1 (200 pg/ml) in the presence of different nominal concentrations of peptide P54, filtered (◆) and unfiltered (•).

Fig. 14. Percentage inhibition of TGFβ1 (200 pg/ml) by receptor peptides derived from modification of peptide P54 (P139 to P143) and of the peptides derived from the human type III receptor (P144 and P145). All the peptides were tested at a concentration of 200 μg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 15. Percentage inhibition of the activity of TGFβ1 (200 pg/ml) in the presence of different nominal concentrations of peptide P144 without filtration.

Fig. 16. Autoradiograph of an affinity labelling test of the receptors of TGFβ1. Lane C1: preincubation was effected with peptide P144 at a concentration 10^6 times greater than the molar concentration of ^{125}I-TGFβ1. Lanes C2 and C3: effect of preincubation of the cells with a concentration of non-radioactive TGFβ1 10 times greater than that of ^{125}I-TGFβ1 (negative control). Lanes C4 and C5: effect of incubation of the cells with a concentration of 0.1 μM of ^{125}I-TGFβ1 that corresponds to an activity of 0.2 μCi (positive control). It can be seen that there is inhibition of the binding of ^{125}I-TGFβ1

to the cell receptors both by peptide P144 and by the non-radioactive TGFβ1.

Fig. 17. Percentage inhibition of TGFβ1 (200 pg/ml) by peptides derived from human type II receptor (P146), from fetuin (P147 to P149) and from endoglin (P150 to P154). All the peptides were tested at a concentration of 200 μg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 18. Percentage inhibition of TGFβ1 (200 pg/ml) by peptides derived from α2-macroglobulin. All the peptides were tested at a concentration of 200 μg/ml. Inhibition of TGFβ1 of 100% corresponds to the growth of MV-1-Lu cells that is obtained in the absence of TGFβ1.

Fig. 19. Percentage inhibition of the binding of TGFβ1 to MV-1-Lu cells by various synthetic peptides. Inhibition was investigated by measuring the percentage of labelled cells (emit fluorescence) and unlabelled cells (do not emit fluorescence) for each peptide (Material and Methods).

Fig. 20. Effect of administration of peptide P144 on collagen synthesis during experimental cirrhosis induction with CCl_4. The ratio of collagen to total protein is shown on the ordinate. The abscissa shows the various groups of rats: Co= healthy rats; Co+P144= healthy rats treated with peptide P144; Tto_1= rats subjected to induction of cirrhosis with CCl_4 and administered peptide P144 on alternate days during this period and Ci_1= rats subjected to induction of cirrhosis with CCl_4 for 11 weeks and not treated with peptide P144.

Fig. 21. Effect of administration of peptide P144 on collagen synthesis during experimental cirrhosis induction with CCl_4. The ordinate shows the ratio of the area of fibrosis to the total area in tissue preparations stained with Sirius Red. The abscissa shows the various groups of rats: Co= healthy rats; Co+P144= healthy rats treated with the peptide; Tto_1= rats subjected to induction of cirrhosis with CCl_4 and administered peptide P144 on alternate days during this period and Ci_1= rats subjected to induction of cirrhosis with CCl_4 for 11 weeks and not treated with peptide P144.

Fig. 22. Effect of administration of peptide P144 on collagen synthesis once cirrhosis has been induced with CCl_4. The ordinate shows the ratio of collagen to total protein. The abscissa shows the various groups of rats: Co= healthy rats; Co+P144= healthy rats treated with the peptide; Tto_2= rats subjected to induction of cirrhosis with CCl_4 and administered peptide P144 on alternate days at the end of this period and Ci_2= rats subjected to induction of cirrhosis with CCl_4 for 11 weeks and not treated with peptide P144.

Fig. 23. Effect of administration of peptide P144 on collagen synthesis once cirrhosis has been induced with CCl_4. The ordinate shows the ratio of the area of fibrosis to the total area in tissue preparations. The abscissa shows the various groups of rats: Co= healthy rats; Co+P144= healthy rats treated with the peptide; Tto_2= rats subjected to induction of cirrhosis with CCl_4 and administered peptide P144 on alternate days at the end of this period and Ci_2= rats subjected to induction of cirrhosis with CCl_4 for 11 weeks and not treated with peptide P144.

Fig. 24. Comparison of the data on quantity of collagen and area of fibrosis, obtained by the two techniques employed. The abscissa shows the values of the ratio of the area of fibrosis to the total area, obtained by image analysis. The ordinate shows the values of the ratio of µg of collagen to mg of total protein, obtained by spectrophotometric analysis of liver sections stained with Direct Red and Fast Green. R^2 is shown. ($F \leq 0.001$).

Fig. 25. Comparison of the data on quantity of collagen and area of fibrosis, obtained by the two techniques employed for examining the samples at the end of protocol 2. The abscissa shows the values of the ratio of the area of fibrosis to the total area, obtained by image analysis. The ordinate shows the values of the ratio of µg of collagen to mg of total protein, obtained by spectrophotometric analysis of liver sections stained with Direct Red and Fast Green. R^2 is shown. ($F \leq 0.001$).

Fig. 26. Images that are representative of the 24 fields obtained by light microscopy (10X) from rat liver preparations stained with Sirius Red. Cirrhotic rats (Ci_1) at the end of induction of cirrhosis with CCl_4 and cirrhotic rats treated (Tto_1) with peptide P144 during induction of cirrhosis with CCl_4. Different fields were taken from preparations obtained from each animal (R= rat and C= field).

Fig. 27. Images that are representative of the 24 fields obtained by light microscopy (10X) from rat liver preparations stained with Sirius Red. Cirrhotic rats (Ci_1) at the end of induction of cirrhosis with CCl_4 and cirrhotic rats treated (Tto_1) with peptide P144

during induction of cirrhosis with CCl_4. Different fields were taken from the preparations obtained from each animal (R= rat and C= field). Polarized light and a green filter were used in order to show up the collagen fibres.

Fig. 28. Comparison between the two groups of untreated cirrhotic rats. Ci_1 are cirrhotic rats at the end of the 12 weeks of induction of cirrhosis with CCl_4, Ci_2 are cirrhotic rats at 4 weeks from the end of the process of induction of cirrhosis. P = 0.016. Ordinate: Area of fibrosis/Total area.